

Comparative Estrogenic Activity of Wine Extracts and Organochlorine Pesticide Residues in Food

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The human diet contains industrial-derived, endocrine-active chemicals and higher levels of naturally occurring compounds that modulate multiple endocrine pathways. Hazard and risk assessment of these mixtures is complicated by nonadditive interactions between different endocrine-mediated responses. This study focused on estrogenic chemicals in the diet and compared the relative potencies or estrogen equivalents (EQs) of the daily consumption of xenoestrogenic organochlorine pesticides in food (2.44 µg/day) with the EQs in a single 200-ml glass of red cabernet wine. The reconstituted organochlorine mixture contained 1,1,1-trichloro-2-(*p*-chlorophenyl)-2-(*o*-chlorophenyl)ethane, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene, endosulfan-1, endosulfan-2, *p,p'*-methoxychlor, and toxaphene; the relative proportion of each chemical in the mixture resembled the composition reported in a recent U.S. Food and Drug Administration market basket survey. The following battery of *in vitro* 17β-estradiol (E₂)-responsive bioassays were utilized in this study: competitive binding to mouse uterine estrogen receptor (ER); proliferation in T47D human breast cancer cells; luciferase (Luc) induction in human HepG2 cells transiently cotransfected with C3-Luc and the human ER, rat ER-α, or rat ER-β; induction of chloramphenicol acetyltransferase (CAT) activity in MCF-7 human breast cancer cells transfected with E₂-responsive cathepsin D-CAT or creatine kinase B-CAT plasmids. For these seven *in vitro* assays, the calculated EQs in extracts from 200 ml of red cabernet wine varied from 0.15 to 3.68 µg/day. In contrast, EQs for consumption of organochlorine pesticides (2.44 µg/day) varied from nondetectable to 1.24 ng/day. Based on results of the *in vitro* bioassays, organochlorine pesticides in food contribute minimally to dietary EQ intake. — *Environ Health Perspect* 106(Suppl 6):1347–1351 (1998). <http://ehpnet1.niehs.nih.gov/docs/1998/Suppl-6/1347-1351gaido/abstract.html>

Key words: estrogenic activity, bioassays, wine extracts, organochlorine pesticide mixtures

Endocrine-active chemicals define a broad class of natural and synthetic chemicals that modulate various constitutive endocrine pathways that are essential for maintaining homeostasis in living organisms. An endocrine-active compound may affect a specific receptor-mediated endocrine pathway by directly binding to

the receptor or by modulating one or more downstream receptor-mediated events. In recent years there has been scientific, regulatory, and public concern over the potential adverse environmental and human health effects of industrial chemicals that bind to the estrogen receptor (ER), androgen receptor, and aryl hydrocarbon

receptor (1–5). Many of the more important classes of persistent organochlorine (OC) pollutants bind to these three receptor systems (6,7) and it has been hypothesized that some of these compounds may be responsible for reproductive problems in wildlife, decreased male reproductive capacity, and breast cancer in women (1–5). The validity of these hypotheses has been questioned (8–10) and ongoing research will help resolve these complex issues.

Environmental estrogens or xenoestrogens have been a major focal point of concern because *in utero* exposure to estrogenic compounds such as the drug diethylstilbestrol can adversely affect both male and female offspring; moreover, lifetime estrogen exposure is a known risk factor for breast cancer in women (11,12). The human diet contains a highly complex mixture of different endocrine-active chemicals including estrogenic flavonoids, lignans, sterols, and fungal metabolites in vegetables, fruits, nuts, and grain-derived products (13–15). Levels of xenoestrogens in the diet have not been fully described; however, at least seven OC contaminants have been identified in the U.S. Food and Drug Administration (U.S. FDA) market basket survey (16) and these include 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (*p,p'*-DDE), 1,1,1-trichloro-2-(*p*-chlorophenyl)-2-(*o*-chlorophenyl)ethane (*o,p'*-DDT), *p,p'*-methoxychlor, endosulfan-1, endosulfan-2, toxaphene, and 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (*p,p'*-DDT). The daily intake of this pesticide mixture is approximately 2.44 µg/day. The estrogenic activity of these compounds has been confirmed in some assays (6,7); however, the effects of reconstituted mixtures of OC pesticides (OC mix) have not been investigated. This study compared the *in vitro* estrogenic activity of naturally occurring estrogens in two wine extracts with a reconstituted mixture of OC pesticides in food using several estrogen-responsive bioassays. A limitation of the reconstituted mixture of pesticides is that it contains only those compounds previously identified as estrogens. It is possible that other contaminants may also exhibit estrogenic activity. The mixtures exhibited a range of estrogenic potencies in these *in vitro* bioassays, and estrogen equivalents in one 200-ml glass of red wine were significantly higher than observed for the estimated daily intake of the OC mix.

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Abbreviations used: CAT, chloramphenicol acetyltransferase; CATH, cathepsin D; CKB, creatine kinase B; *p,p'*-DDE, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene; *o,p'*-DDT, 1,1,1-trichloro-2-(*p*-chlorophenyl)-2-(*o*-chlorophenyl)ethane; *p,p'*-DDT, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane; E₂, 17β-estradiol; EQs, estrogen equivalents; ER, estrogen receptor; hER, human estrogen receptor-α; LH, luteinizing hormone; Luc, luciferase; OC, organochlorine; OC mix, reconstituted mixtures of organochlorine pesticides; U.S. FDA, U.S. Food and Drug Administration.

Materials and Methods

Chemicals

o,p'-DDT, *p,p'*-DDT, and *p,p'*-DDE were obtained from Geigy Agricultural Chemicals (Novartis, Greensboro, North Carolina) and were 99.4, 99.9, and 99.2% pure, respectively; *p,p'*-methoxychlor, endosulfan-1, and endosulfan-2 were purchased from Chem-Services (West Chester, Pennsylvania) and were >99% pure. Toxaphene was a reference standard provided by the U.S. Environmental Protection Agency (Research Triangle Park, North Carolina). The OC mix contained the following compounds (percent by weight): *o,p'*-DDT (0.03), *p,p'*-DDT (1.9), *p,p'*-DDE (41.3), *p,p'*-methoxychlor (34.2), endosulfan-1 (4.6), endosulfan-2 (7.2), and toxaphene (10.8), respectively. This reconstituted mixture resembled the relative composition of OC xenoestrogens determined in a 1995 to 1996 U.S. FDA market basket survey for contaminants in food in which average daily intake was 2.44 μg (16). California white chablis and red cabernet wines and a blended American whiskey were purchased locally.

The wine and whiskey extracts were prepared using the following extraction methods: The alcohol beverage (200 ml) was evaporated to dryness *in vacuo* at $\leq 60^\circ\text{C}$; the residue was then resuspended in 200 ml methanol and stirred vigorously for 4 to 6 hr at 20°C . The resulting mixture was

filtered to remove solid debris and the methanol extract was evaporated to dryness. The final extraction utilized ethanol:chloroform (15:85, 100 ml) and the mixture was vigorously stirred for 12 to 18 hr. The ethanol-chloroform extract was filtered, evaporated to dryness, and redissolved in 2 ml water:ethanol (85:15 by volume) buffered with 0.05 M sodium bicarbonate. These extracts were also diluted in the same aqueous ethanol buffer and used in the bioassays. All other chemicals and biochemicals were of the highest quality available from commercial sources. [^{14}C]Chloramphenicol (53 mCi/mmol) was purchased from NEN Research Products (Boston, Massachusetts) and [^3H]17 β -estradiol (E_2) (130 Ci/mmol) was purchased from Amersham Life Sciences (Arlington Heights, Illinois).

Bioassays for Estrogenic Activity

The bioassays utilized in this study have previously been reported (17–23) and include ER binding using B6C3F₁ mouse uterine cytosol; MCF-7 and T47D cell proliferation (for 14 days); induction of chloramphenicol acetyltransferase (CAT) activity in MCF-7 cells transiently transfected with plasmid cathepsin D (pCATH)-CAT and pCKB-CAT; and induction of luciferase (Luc) activity in HepG2 cells transiently cotransfected with complement C3-Luc and an ER expression plasmid. The human CATH construct contains a

promoter insert (–365 to –10) (24) ligated into a pBL/TATA/CAT plasmid derived from pBL/CAT2. The promoter region was derived from a construct originally provided by A. Hasilik (University of Muenster, Muenster, Germany). The creatine kinase B (CKB)-CAT construct contains a 2.9-kb region from the rat CKB gene promoter and was provided by P. Benfield (Dupont Corp., Wilmington, Delaware) (25). Rat ER- α and ER- β expression plasmids were obtained from R. Day (University of Virginia, Charlottesville, Virginia) and J.-A. Gustafsson (Karolinska Institute, Huddinge, Sweden), respectively; D. McDonnell (Duke University, Durham, North Carolina) provided the human ER- α (hER) expression plasmid.

Results

The results illustrated in Figure 1 show that unlabeled E_2 and the chablis and cabernet wine extracts competitively displaced [^3H] E_2 from the mouse uterine ER, whereas the whiskey extract and the highest concentration of the OC mix did not significantly displace the radiolabeled hormone. The whiskey extract was inactive in all of the assay systems and additional results for this extract are not presented in this study. The effects of the wine extracts and the OC mix on proliferation of ER-positive T47D breast cancer cells were determined in this study using assay procedures previously described (23). The results in Figure 2A demonstrate

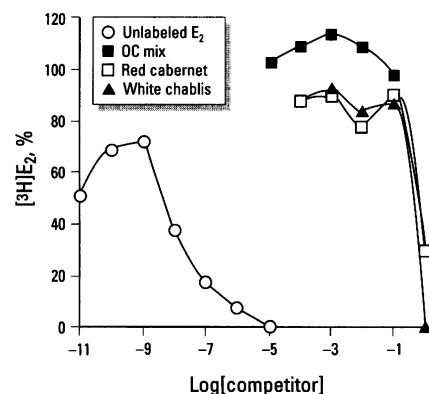


Figure 1. Competitive binding of organochlorine pesticide mixture, two wine extracts, and unlabeled 17 β -estradiol to the mouse cytosolic estrogen receptor. Uterine ER was incubated with [^3H] E_2 and different concentrations of unlabeled E_2 , OC pesticides, red cabernet, and white chablis wine extracts, and the displacement of radiolabeled hormone was determined as previously described by Ramamoorthy et al. (22). The unlabeled E_2 and both wine extracts competitively displaced [^3H] E_2 from the ER, whereas the OC mix and a whiskey extract (data not shown) were inactive.

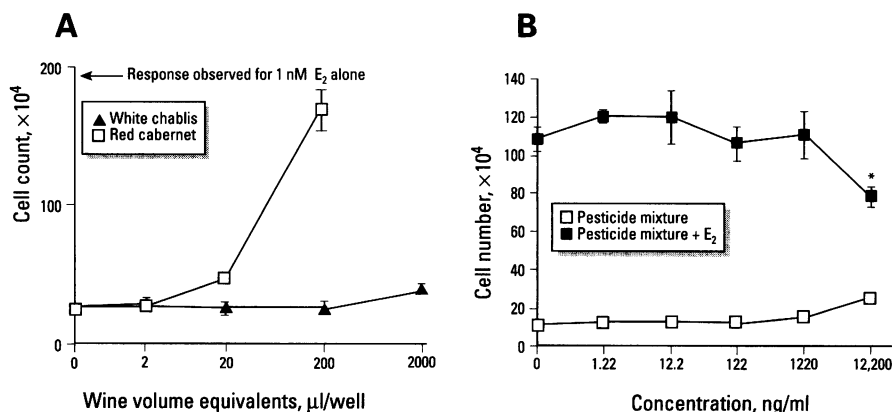


Figure 2. Effects of 17 β -estradiol and various mixtures on proliferation of T47D cells. Cells were treated with (A) 10^{-9} M E_2 , different dilutions of red cabernet or white chablis wine extracts for 14 days or (B) different concentrations of OC pesticides alone or in combination with 10^{-9} M E_2 for 14 days as previously described for MCF-7 (22) and T47D (23) cells. Results are presented as means of three replicate experiments. Significant ($p < 0.05$) induction of cell growth was observed for E_2 , the red cabernet (200 μl wine-equivalents/well), and the white chablis (2000 μl wine-equivalents/well). The response observed for 1n ME_2 alone is illustrated in A. The OC mix increased cell proliferation and inhibited E_2 -induced cell proliferation only at the highest concentration. With the exception of methoxychlor, the remaining compounds alone or in combination with E_2 exhibited minimal activity. The weak ER agonist/partial antagonist response observed for the mixture was significant only for the inhibitory response ($*p < 0.05$) and similar to the effects of methoxychlor (data not shown).

that the highest concentration of the red cabernet wine induced a near maximal cell proliferation response (observed for 1 nM E_2), whereas lower responses were observed for the chablis and the OC mix (Figure 2B). Similar results were obtained in MCF-7 cells. In T47D cells cotreated with 1 nM E_2 and different concentrations of the OC mix, an antiestrogenic response was observed (Figure 2B). Similar results were obtained with the chablis wine, whereas the more highly estrogenic cabernet wine extract was not antiestrogenic in this assay (data not shown).

The results summarized in Figure 3 also show that the red cabernet was significantly more estrogenic than the chablis wine or the OC mix in MCF-7 cells transiently transfected with pCKB-CAT or pCATH-CAT. These results complement the effects of these mixtures on proliferation of T47D (Figure 2) and MCF-7 cells (data not shown).

The results in Figure 4 summarize the effects of the individual OC pesticides and the OC mix on induction of Luc activity in HepG2 cells transiently cotransfected with hER (human), ER- α (rat), and ER- β (rat) expression plasmids. The results show that all of the OC pesticides and the OC mix were active in the HepG2 cell assays. There were only minor differences in activities of individual pesticides using the different ER expression plasmids. The HepG assay system was more sensitive to the estrogenic

activity of the OC pesticides and reconstituted mixture than the other *in vitro* assays used in this study (Figures 1–3) or in the yeast-based assay system (18). The estrogen equivalents (EQs) for the highest concentration of the OC mix and red cabernet wine extract could be estimated by comparing their estrogenic activity to that observed for E_2 alone. The EQ values derived from all assays for extracts of red wine (200 ml) varied from 0.15 to 3.68 μ g/day, whereas values for the OC mix were < 1.24 ng/day (Table 1).

Discussion

The human diet contains relatively high levels of estrogenic compounds, particularly the bioflavonoids, which are ubiquitous in fruits, nuts, vegetables, and grain products. Kuhnau (13) estimated that the average daily intake of flavonoids is approximately 1 g per day; however, only a fraction of this total would constitute estrogenic compounds. Consumption of natural estrogenic compounds is high in Far Eastern countries in which high levels of soy-based products are an important part of the diet. Setchell and co-workers (26) recently reported that 4-month-old infants on soy-based formula consume over 40 mg of total soy-based isoflavones per day. Moreover, plasma levels of estrogenic isoflavones in adults and infants who consume soy foods and soy infant formula can be as high as 10^5 to 10^6 pg/ml.

Gavaler and co-workers (27–33) have previously investigated the estrogenic activity of bourbon and bourbon extracts in both *in vivo* and *in vitro* models. Bourbon extracts contain estrogenic flavonoids and sterols that bind to the ER, and after administration to ovariectomized rats, there was an increase in uterine wet weight and decreased plasma luteinizing hormone (LH) levels. These data clearly demonstrate an *in vivo* estrogenic response. The estrogenic activity of bourbon extracts was also confirmed in clinical studies in which bourbon extracts (equivalent to greater than three drinks/day) were administered to four postmenopausal women for 28 days. LH and follicle-stimulating hormone levels decreased and prolactin, high-density lipoprotein cholesterol, and steroid hormone-binding globulin levels increased during the treatment but returned to background levels after 5 weeks (1 week postexposure). It was also reported that white chablis and red cabernet wines also competitively bound to the ER, and similar results were obtained in the present study (Figure 1). In contrast, the OC mix did not competitively bind to the mouse ER and therefore the comparative estrogenic potency of wine extracts and the OC mix were investigated in multiple assays.

Results of this study demonstrate that the red cabernet wine extract was active in all bioassays whereas with the exception of the ER-binding assay, white wine extracts exhibited lower estrogenic activity than the red cabernet, and the whiskey extracts were inactive. The OC mix exhibited estrogenic activity in the HepG2 cell assay but was inactive or only minimally active in the cell proliferation, ER-binding, and transient transfection assays in MCF-7 cells. The E_2 equivalents could be calculated for the highest concentrations of the red cabernet wine extract and OC mix for each assay by estimating the concentration of E_2 required to induce the same response (assuming a linear E_2 dose-response curve). This approach was utilized to compare EQs for wine extracts and the OC mix in seven E_2 -responsive assays and to calculate daily EQs for the OC mix (2.44 μ g) and a glass of red wine (200 ml). The results obtained for ER binding, T47D cell proliferation, and induction of CAT activity in MCF-7 cells transiently transfected with CKB-CAT or CATH-CAT constructs indicate that EQs associated with a 200-ml glass of wine varied from 0.15 to 0.6 μ g/day, whereas the OC mix was inactive or gave minimal EQ values in these assays (Table 1). In contrast, both wine extracts and the OC mix were active in the

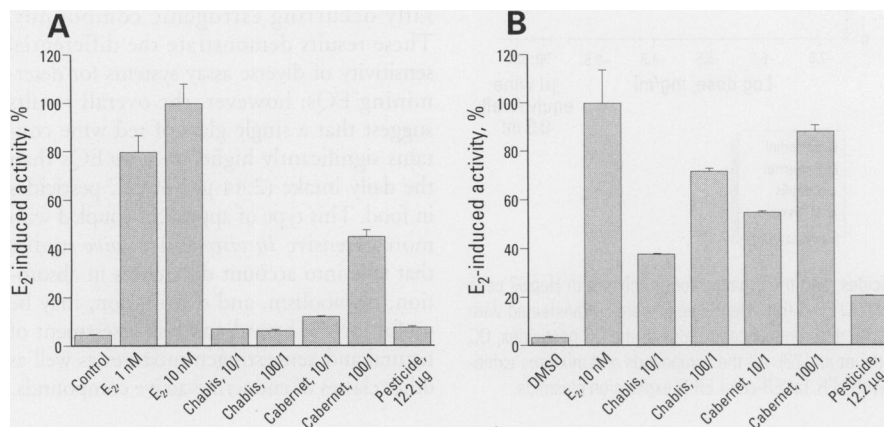


Figure 3. Induction of chloramphenicol acetyltransferase activity in MCF-7 cells after treatment with 17 β -estradiol, the organochlorine pesticide mixture, and wine extracts. DMSO, dimethylsulfoxide. MCF-7 cells were transiently transfected with 4 or 5 μ g hER and 10 μ g CKB-CAT or 5 μ g CATH-CAT constructs and treated with various mixtures. CAT activity was determined as described (22). The designations of 10/1 and 100/1 are concentration factors for the reconstituted wine extracts and represent the equivalents of 1 and 10 ml of wine added to each plate, which contains 10 ml media. The highest concentration of the OC mix was 12.2 μ g/ml. Results are expressed as means \pm SE for three separate experiments. The red wine extracts significantly induced CAT activity in MCF-7 cells transiently transfected with (A) CATH-CAT or (B) CKB-CAT constructs, whereas the white wine extracts exhibited lower activity and the OC mix was inactive.

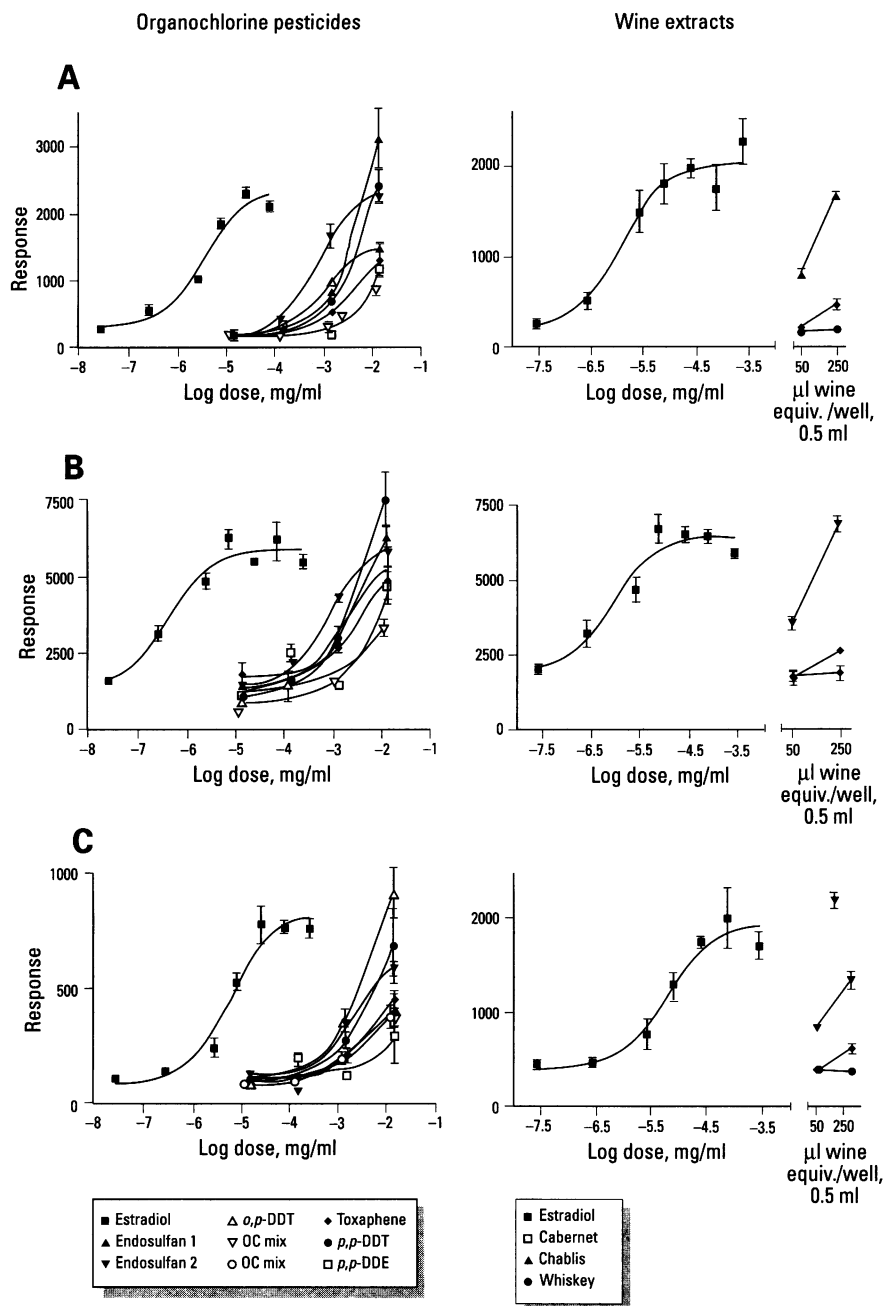


Figure 4. Estrogenic activity of wine extract, individual pesticides, and the organochlorine mixture in HepG2 cells cotransfected with (A) variable hER (human), (B) ER- α (rat), and (C) ER- β (rat). HepG2 cells were cotransfected with C3-Luc and various ER expression plasmids and then treated with different concentrations of the OC pesticides, OC mix, and wine extracts as previously described by Ramamoorthy et al. (22). All the compounds and mixtures exhibited estrogenic activity in HepG2 cells transiently transfected with hER, rat ER- α , or ER- β expression plasmids.

Table 1. A comparison of daily 17 β -estradiol equivalents in a glass of red cabernet wine and organochlorine pesticides in food.^a

Assay	Sample	Estimated daily intake of E ₂ equivalents, μ g
ER binding	Wine	0.6
	OC mix	ND
Cell proliferation	Wine	0.45
	OC mix	<10 ⁻⁵
CAT activity (CD)	Wine	0.24
	OC mix	ND
CAT activity (CKB)	Wine	0.15
	OC mix	ND
HepG2-ER human	Wine	1.87
	OC mix	0.00021
HepG2-ER- α rat	Wine	0.94
	OC mix	0.00072
Hep G2-ER- β rat	Wine	3.68
	OC mix	0.0012

ND, nondetectable. ^aThese experiments were performed as previously described by Ramamoorthy et al. (17,18,22), Gaido et al. (19), Tzukerman et al. (20), McDonnell et al. (21), and Fernandez and Safe (23) and E₂ equivalents for the 200-ml red cabernet and 2.44 μ g/day OC mix were calculated for each bioassay. The whiskey extract was not estrogenic and the EQ for the 200-ml white chablis was at least 10 times lower than the values obtained for the red cabernet.

HepG2 cell assay and their corresponding EQ values varied from 0.94 to 3.68 and from 0.00072 to 0.0012 μ g/day, respectively. This study only estimates EQ values for intakes of the estrogenic mixtures and does not take into account body burdens or serum levels of OC pesticides or other naturally occurring estrogenic compounds. These results demonstrate the differential sensitivity of diverse assay systems for determining EQs; however, the overall results suggest that a single glass of red wine contains significantly higher *in vitro* EQs than the daily intake (2.44 μ g) of OC pesticides in food. This type of approach, coupled with more extensive *in vitro* and *in vivo* studies that take into account differences in absorption, metabolism, and distribution, may be useful for the hazard and risk assessment of natural and xenoestrogen mixtures as well as other classes of endocrine-active compounds.

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